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Interactions of angiotensin II non-peptide AT_1 antagonist losartan with phospholipid membranes studied by combined use of differential scanning calorimetry and electron spin resonance spectroscopy

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Abstract

We used differential scanning calorimetry (DSC) and electron spin resonance (ESR) spectroscopy to investigate the interactions of Losartan, a potent, orally active Angiotensin II AT_1 receptor antagonist with phospholipid membranes. DSC results showed that Losartan sensitively affected the chain-melting behavior of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) bilayer membranes. ESR spectroscopy showed that phosphatidylcholines spin-labeled at the 5-position of the *sn*-2 acyl chain (*n*-PCSL with $n = 5$), incorporated either in DMPC or DPPC bilayers containing Losartan, were restricted in motion both in the gel and in the liquid–crystalline membrane phases, indicating a location of the antagonist close to the interfacial region of the phosphatidylcholine bilayer. At high drug concentrations (mole fraction $\geq x = 0.60$), the decrease in chain mobility registered by 5-PCSL in fluid-phase membranes is smaller than that found at lower concentrations, whereas that registered by 14-PCSL is further increased. This indicates a different mode of interaction with Losartan at high concentrations, possibly arising from a location deeper within the bilayer. Additionally, Losartan reduced the spin–spin broadening of 12-PCSL spin labels in the gel-phase of DMPC and DPPC bilayers. As a conclusion, our study has shown that Losartan interacts with phospholipid membranes by affecting both their thermotropic behavior and molecular mobility.

Keywords: Angiotensin II; Losartan; Phospholipid membrane; Differential scanning calorimetry; Electron spin resonance spectroscopy

1. Introduction

Angiotensin II (ANG II) is an endogenous octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) produced by the renin–angiotensin system. This hormone is the body's most potent vasoconstrictor. It affects the blood pressure directly by constriction of the blood vessels and, indirectly, by inducing the release of aldosterone from the cortex of the adrenal gland.

Besides its action as a vasoconstrictor, ANG II is involved in many biological effects and it has a wide

Abbreviations: Losartan, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole potassium salt; ANG II, angiotensin II; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; *n*-PCSL, 1-acyl-2-[*n*-(4,4′-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; ESR, electron spin resonance

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range of target tissues [1]. It induces the release of catecholamines in the adrenal medulla and the synthesis of prostaglandins in the kidney. Additionally, it stimulates ovulation, and glycogenolysis in the liver. In the brain, ANG II induces the release of vasopressin and the release of the corticotropin hormone (ACTH) in the hypophysis.

ANG II exerts its biological function by interacting with specific cellular receptors found in the membranes of many tissues. This interaction activates a messenger system which finally leads to the biological response [2]. Two major subtypes of receptors, designated as AT_1 and AT_2, have been identified in a variety of animal and human tissues [3,4].

The major physiological functions of ANG II have hitherto been attributed to the G-protein-coupled AT_1 receptor [5]. Therefore, antagonism of ANG II at its receptor by biologically active molecules would be the most selective method for the treatment of hypertension [6]. Since the discovery of non-peptide ANG II receptor antagonists in 1982 by Takeda [7], a large number of such antagonists has been synthesized [8–10].

Losartan (Fig. 1) is a potent and selective orally active non-peptide receptor antagonist, developed jointly by the Du Pont (DUP 753) and Merck (MK 954) companies and later distributed solely by MSD (COZAAR or LORZAAR), which recently received FDA approval as a new drug [11,12].

Existing evidence suggests that ANG II also interacts with membranes. Radioimmunoassays and receptor-binding assays revealed the interference of ANG II interactions by some lipids [13,14]. It is also known that ANG II is able to mediate the transport of Mn^{2+} across phosphatidylcholine bilayers [15]. Binding interactions between human ANG II and dipalmitoylphosphatidylcholine (DPPC) vesicles have been detected by measuring the selective proton spin-lattice relaxation rates of aromatic protons within the peptide [16]. Recently, by using a combination of ^{31}P-NMR spectroscopy and differential scanning calorimetry (DSC), we showed that ANG II affects the phase properties of DPPC bilayers and produces effects on the phospholipid head groups [17]. However, the interactions of non-peptide AT_1 receptor antagonists with membranes have not been studied in detail. In the present work, we used high resolution DSC and electron spin resonance (ESR) spectroscopy to investigate the interaction of Losartan, the AT_1 receptor antagonist, with phosphatidylcholine bilayers. Phosphatidylcholine is the most important structural class of mammalian membrane phospholipid.

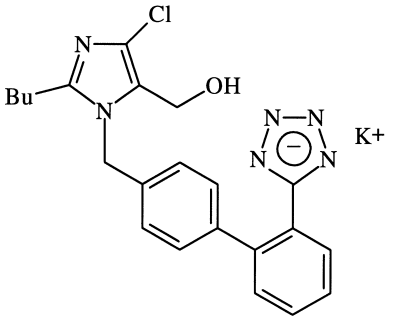

Fig. 1. Chemical structure of Losartan potassium salt.

DSC is a thermodynamic technique which allows us to investigate the thermotropic properties of membranes in the absence and presence of biologically active molecules [18–21]. Spin label ESR spectroscopy has been used extensively in studies of lipid chain dynamics and of the molecular location of proteins in phospholipid bilayers [22–25].

2. Materials and methods

2.1. Materials

Dimyristoylphosphatidylcholine (DMPC) and dipalmitoyl-phosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids (Birmingham, Alabama, USA). Losartan was kindly provided by Merck's Research Laboratories (MSD Sharp and Dohme, Haar, Germany). Phospholipid spin labels (*n*-PCSL) were synthesized by B. Angerstein as described in [26].

2.2. Differential scanning calorimetry

Thermograms of hydrated mixtures of phospholipids with Losartan at molar ratios of 99:1 ($x=0.01$), 95:5 ($x=0.05$), 90:10 ($x=0.10$), 80:20 ($x=0.20$), 60:40 ($x=0.40$) and 50:50 ($x=0.50$) of lipid:drug were obtained.

The preparation of DSC samples was identical for the two phospholipids used in this study. A 7-mg

amount of the phospholipid and appropriate amounts of Losartan were codissolved in a dichloromethane/methanol mixture (2:1, v/v). The organic solvent was evaporated under a stream of oxygen-free N_2, at a temperature above the corresponding phase transition temperature of the phospholipid. The lipid/drug mixtures were then dried under high vacuum overnight.

DSC was performed with a Model 4207 heat-flow calorimeter from Hart Scientific (Pleasant Grove, Utah). The dried samples were hydrated with 200 μl double distilled and deionized water and transferred to the DSC ampoules. Distilled and deionized water was used as a reference sample. Prior to scanning, the samples were held above their phase transition temperature for 10 min to ensure complete hydration. All samples were scanned at least twice until identical thermograms were obtained using a scan rate of 10°C/h. Integration of the calorimetric endotherms was performed using the software supplied with the instrument.

2.3. Electron spin resonance spectroscopy

Dried mixtures of phospholipids and Losartan were prepared following the procedure described above. In the case of samples for ESR spectroscopy, 1 mg of the phospholipid and appropriate amounts of the antagonist were used and 1 mol% of the spin-labeled lipid in dichloromethane was added.

After hydration with 50 μl distilled and deionized water, the samples were transferred to ESR capillaries of 1 mm diameter and concentrated by centrifugation (10 000 rpm, for 30 min at 4°C). ESR spectra were obtained on a Varian E-12 Century Line 9 GHz spectrometer. ESR capillaries were inserted in a standard 4-mm quartz ESR tube containing light silicone oil for thermal stability. A pure nitrogen gas-flow system was used to regulate the temperature. ESR spectra were recorded using a modulation frequency of 100 kHz, modulation amplitude of 1.25 G peak-to-peak and total scan width 100 G.

Outer hyperfine splittings ($2A_{max}$) were used to characterize the rotational disorder and rotational rates of the spin-labeled lipid chain segments [27]. This quantity is defined on the experimental spectra given subsequently (Fig. 4). In general, the values of $2A_{max}$ will depend both on the order parameter of the spin-labeled chain segment and on rotational correlation time. For fluid-phase membranes, it will depend most strongly on the chain order, but in gel-phase membranes, it will also depend strongly on the (slow) rotational rates. For rotational mobility with components in the slow-motional regime [28], the change in this parameter, ΔA_{max}, therefore provides a useful measure of the change in lipid chain mobility induced by Losartan. Rapid isotropic rotation reduces A_{max} to $A_0 = 15.0$ G, which is the isotropic average hyperfine coupling constant [25]. Therefore, $\Delta A_{max}/(A_{max} - A_0)$ is the appropriate normalized quantity that characterizes the changes in A_{max}.

3. Results

3.1. Differential scanning calorimetry

The DSC traces of DMPC preparations containing increasing concentrations of Losartan are shown in

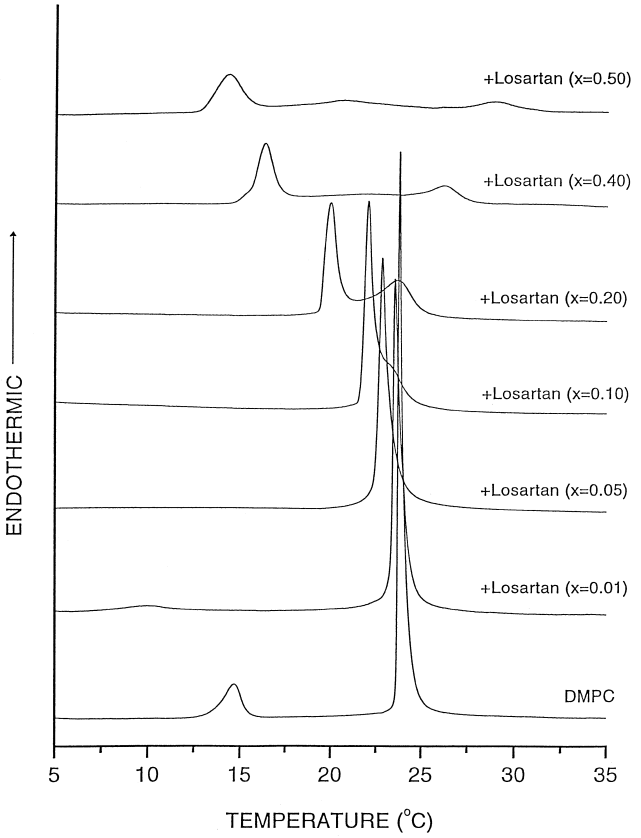

Fig. 2. DSC thermograms of hydrated DMPC bilayers containing Losartan at the different mole fractions, x, indicated.

Table 1
Calorimetric peak temperatures (T_c), half-widths ($\Delta T_{c1/2}$) and total enthalpy changes (ΔH) of DMPC bilayers containing different concentrations of Losartan

Sample	T_c (°C)[a]		ΔH (kcal/mol)[a]		$\Delta T_{c1/2}$ (°C)[a]	
DMPC	14.7	23.8	1.0	5.3	1.2	0.3
+Losartan ($x=0.01$)	10.0	23.5	0.4	5.2	2.6	0.5
+Losartan ($x=0.05$)	–	22.9	–	5.0	–	0.6
+Losartan ($x=0.10$)	–	22.1/23.2[b]	–	4.8	–	0.5
+Losartan ($x=0.20$)	–	20.0/23.7	–	5.5	–	0.8/2.6[b]
+Losartan ($x=0.40$)	–	16.3/26.2	–	5.7	–	1.0/2.2
+Losartan ($x=0.50$)	–	14.3/29.0	–	6.7	–	1.8/3.1

[a]Values to the left of each column correspond to the pretransition and those to the right to the main phase transition.
[b]Double values refer to the two outer peaks in the total endotherm.

Fig. 2. The hydrated pure DMPC shows a pretransition centered at 14.7°C and a main phase transition from the gel to the liquid crystalline phase at 23.8°C, in agreement with previous reports [29]. The pretransition of DMPC is strongly affected by the presence of the antagonist in the membranes, being broadened, shifted to much lower temperatures and already abolished at low concentration $x=0.05$. At Losartan concentrations up to $x=0.10$, a progressive shift of the main transition to lower temperatures and a broadening of the half width of the corresponding peak are observed, as is shown in Table 1. At mole fraction $x=0.10$, a high temperature shoulder is formed at around 23°C. As the concentration is increased to $x=0.50$, this shoulder becomes a new peak which is shifted to higher temperatures, while the peak on the low temperature side is shifted to lower temperatures. A broader intermediate peak also appears close to the mid-point of the transition region. Increase in the concentration of Losartan therefore causes a large broadening in extent of the transition region (ca. 20°C at $x=0.5$). Concomitantly, the sample becomes highly viscous in the transition region. As shown in Table 1, the presence of the drug does not affect drastically the total enthalpy change (ΔH) of the observed phase transition.

The DSC thermograms of hydrated pure DPPC and mixtures with Losartan are shown in Fig. 3. DPPC bilayers show a pretransition at 34.9°C and a main phase transition from the gel to the liquid crystalline phase at 41.2°C, in agreement with previous reports [29]. The incorporation of increasing concentrations of Losartan results in a progressive shift of the main transition to lower temperatures and a broadening of the corresponding peak (Table 2). The pretransition is again strongly affected and is abolished at low concentration of the antagonist ($x=0.10$). The enthalpy change of the gel to liquid crystalline phase transition of DPPC was not decreased very significantly by the incorporation of Losartan (Table 2).

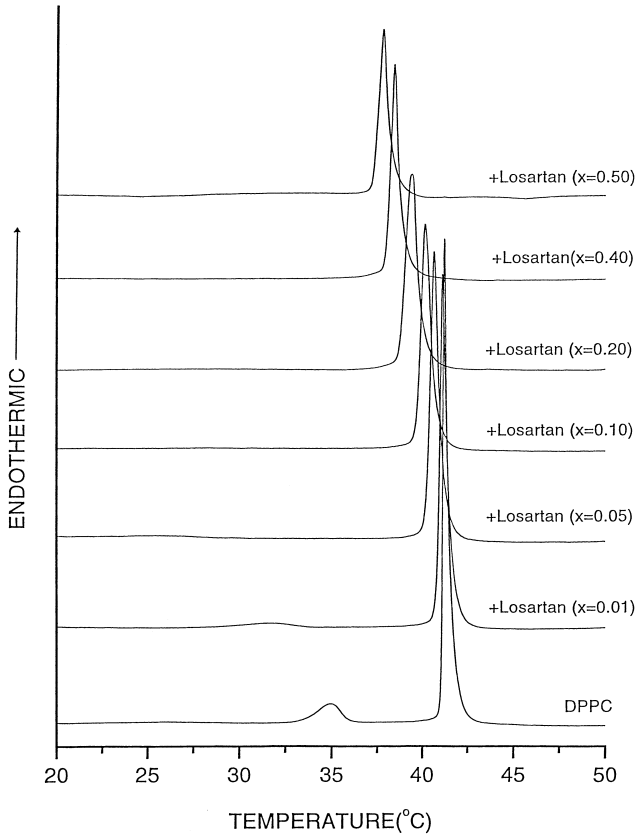

Fig. 3. DSC thermograms of hydrated DPPC bilayers containing Losartan at the different mole fractions, x, indicated.

3.2. Electron spin resonance spectroscopy

ESR spectra of phosphatidylcholines spin-labeled at the 5-, 12- or 14-position of the *sn*-2 chain (5-PCSL, 12-PCSL and 14-PCSL, respectively) incorporated into DMPC bilayers are compared in Fig. 4 (dotted lines) with the corresponding ones from preparations containing Losartan at $x = 0.10$ (solid lines). The spectra were obtained at different temperatures in order to cover all mesomorphic phases of the bilayer membranes.

In the case of 5-PCSL spin labels, DMPC bilayers exhibit a chain-melting phase transition in the region around 23°C, as is observed from the decrease of the outer hyperfine splitting $2A_{max}$ ($\Delta A_{max}/(A_{max}-A_0) = 28.5\%$ between 20 and 25°C). The bilayers containing Losartan exhibit this transition in a region somewhat below 23°C. Additionally, in the preparation containing Losartan, the spin labels are more restricted in their motion in both the gel and liquid–crystalline phases because the outer hyperfine splitting $2A_{max}$ is increased in the presence of Losartan, relative to the spectra in its absence ($\Delta A_{max}/(A_{max}-A_0) = 2.9\%$ and 4.5% at 10°C and 35°C, respectively). As is evident in Fig. 4, the spin–spin broadening of 12-PCSL spin labels in the gel phase of DMPC bilayers is reduced by the presence of Losartan in the membranes. Spin–spin broadening arises from partial exclusion of the spin-labeled phospholipids from the tightly packed DMPC molecules in the gel phase. The reduction of spin–spin broadening that is observed demonstrates that Losartan improves the solubility of the lipid probes in the gel

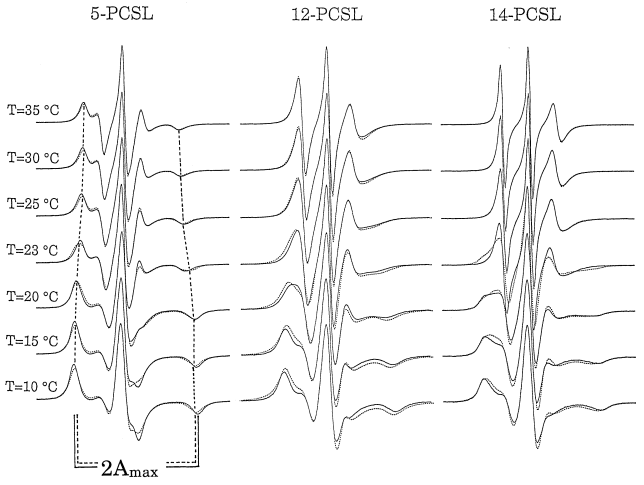

Fig. 4. ESR spectra of 5-, 12- and 14-PCSL spin labels incorporated either in DMPC bilayers (dotted line) or DMPC bilayers containing Losartan at mole fraction $x = 0.10$ (solid line) obtained at the temperatures indicated, over the range 10–35°C. The outer hyperfine splitting $2A_{max}$ for DMPC alone is indicated by dashed lines. Total scan width = 100 G.

Table 2
Transition temperatures (T_c), half-widths ($\Delta T_{c1/2}$) and enthalpy changes (ΔH) of DPPC bilayers containing different concentrations of Losartan

Sample	T_c (°C)[a]		ΔH (kcal/mol)[a]		$\Delta T_{c1/2}$ (°C)[a]	
DPPC	34.9	41.2	1.2	7.6	1.6	0.3
+Losartan ($x = 0.01$)	31.8	41.1	0.6	7.4	3.2	0.4
+Losartan ($x = 0.05$)	25.0	40.6	0.3	7.3	3.9	0.5
+Losartan ($x = 0.10$)	–	40.1	–	7.0	–	0.6
+Losartan ($x = 0.20$)	–	39.4	–	7.3	–	0.7
+Losartan ($x = 0.40$)	–	38.4	–	7.3	–	0.5
+Losartan ($x = 0.50$)	–	37.8	–	7.3	–	0.6

[a]Values to the left of each column correspond to the pretransition and those to the right to the main phase transition.

phase. ESR spectra of 14-PCSL spin labels show that the preparation containing the antagonist undergoes a chain-melting phase transition in the region around 20°C while the corresponding pure lipid preparation undergoes this transition in the region around 23°C. Moreover, in the presence of Losartan, 14-PCSL spin labels are more restricted in motion in the gel phase, relative to in its absence ($\Delta A_{max}/(A_{max}-A_0) = 9.8\%$ at 10°C).

The concentration of the antagonist in DMPC bilayers was increased further to mole fractions $x = 0.40$ and $x = 0.60$ and the corresponding ESR spectra of 5- and 14-PCSL spin labels are shown in Fig. 5. At mole fraction $x = 0.40$ (Fig. 5A), the effects of Losartan are qualitatively similar to those described above for the lower mole fraction $x = 0.10$. Quantitatively, the effects are correspondingly larger. The 5-PCSL spin labels are restricted in motion both in the gel and in the liquid–crystalline phase, showing greater increases in outer hyperfine splitting $2A_{max}$ than at the lower Losartan concentration of $x = 0.10$ ($\Delta A_{max}/(A_{max}-A_0) = 3.0\%$ and 8.3% at 10°C and 35°C, respectively). Furthermore, the observed chain-melting transition is shifted to lower temperatures than at mole fraction $x = 0.10$. ESR spectra of the 14-PCSL spin labels show that the preparation containing the antagonist undergoes chain-melting at

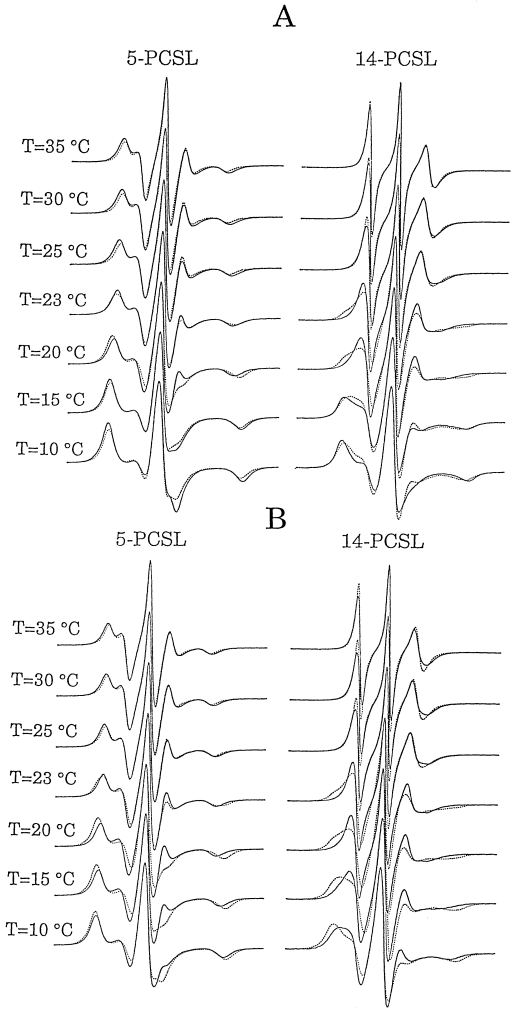

Fig. 5. ESR spectra of 5- and 14-PCSL spin labels incorporated either in DMPC bilayers (dotted line) or DMPC bilayers containing Losartan (solid line) at mole fraction $x = 0.40$ (A) or $x = 0.60$ (B) obtained at the temperatures indicated, over the range 10–35°C. Total scan width = 100 G.

lower temperatures and that the spin probes are more restricted in motion in both the gel and fluid phases, relative to the situation at $x = 0.10$ ($\Delta A_{max}/(A_{max} - A_0) = 10.1\%$ and 22.6% at 10°C and 35°C, respectively).

At yet higher Losartan concentration $x = 0.60$ (Fig. 5B), a further shift and widening of the chain-melting region is induced and, as a result, the spectra of 5-PCSL spin labels show decreased hyperfine splittings $2A_{max}$ in the gel phase compared to the corresponding ones of spin labels incorporated into pure DMPC ($\Delta A_{max}/(A_{max} - A_0) = 3.7\%$ at 10°C). Although a restriction in motion of the spin probes is seen in the fluid phase, the increase observed in outer hyperfine splittings is smaller than the one evidenced in the spectra of the preparation containing Losartan at mole fraction $x = 0.40$ ($\Delta A_{max}/(A_{max} - A_0) = 5.3\%$ as opposed to 8.3% at 35°C). In the case of the 14-PCSL spin labels, the shift and broadening of the chain-melting region is also seen in the gel phase, while in the fluid phase, a marked decrease in acyl chain motion of the spin probes, relative to $x = 0.40$, is observed ($\Delta A_{max}/(A_{max} - A_0) = 73.6\%$ as opposed to 22.6% at 35°C).

Losartan was incorporated also into DPPC bilayers at a mole fraction $x = 0.10$, and the corresponding ESR spectra of 5-, 8-, 12- and 14-PCSL spin labels are shown in Fig. 6. It is observed that the 5-PCSL spin label is motionally restricted both in the gel and in the liquid–crystalline phase due to the presence of Losartan in the membranes ($\Delta A_{max}/(A_{max} - A_0) = 3.1\%$ and 3.0% at 30°C and 43°C, respectively). Moreover, Losartan shifts the observed chain-melting transition to between 38 and 40°C, while pure DPPC bilayers exhibit this transition between 41 and 43°C. A clear reduction of spin–spin broadening on incorporation of the antagonist in DPPC bilayers is observed in the gel phase for the ESR spectra of the 8- and 12-PCSL spin labels, and to a lesser extent for the 5- and 14-PCSL spin labels. Additionally, Losartan shifts the chain-melting transition recorded by the 8-, 12- and 14-PCSL spin labels to lower temperature (between 38 and 40°C). It is also observed that the 8- and 12-PCSL spin labels are restricted in motion by the antagonist in the gel phase ($\Delta A_{max}/(A_{max} - A_0) = 4.9\%$ and 6.3%, respectively at 20°C).

ESR spectra of 5- and 14-PCSL spin labels incorporated in DPPC membranes containing Losartan at mole fractions of $x = 0.40$ and $x = 0.60$ are shown in Fig. 7. At drug concentration $x = 0.40$ (Fig. 7A), a further shift of the chain-melting transition to around 34–36°C is observed in the spectra of the 5-PCSL spin label. Additionally, the 5-PCSL spin label is motionally restricted in both the gel and fluid phases, showing greater increases in outer hyperfine splitting $2A_{max}$ than at the lower Losartan mole fraction $x = 0.10$ ($\Delta A_{max}/(A_{max} - A_0) = 5.3\%$ and 3.9% at 30°C and 43°C, respectively). Furthermore, ESR spectra of the 14-PCSL spin labels show that the spin probes are more restricted in motion

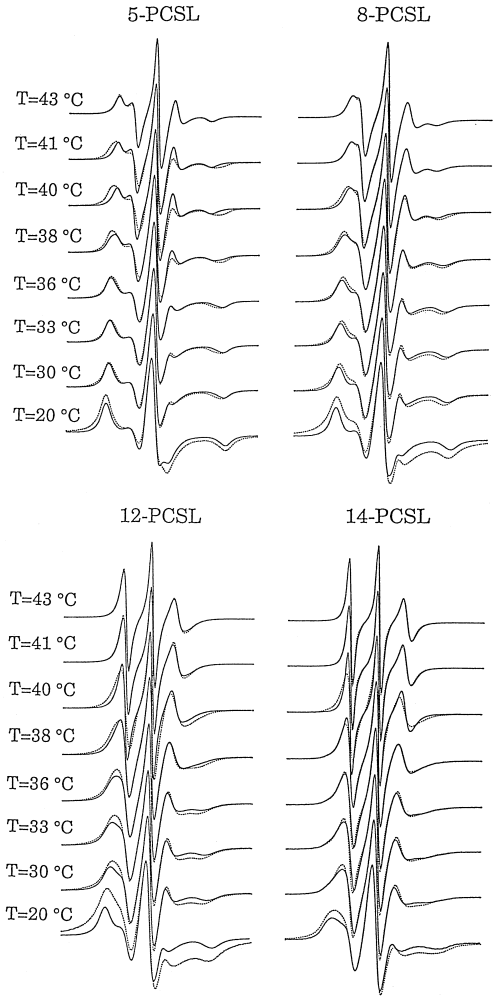

Fig. 6. ESR spectra of 5-, 8-, 12- and 14-PCSL spin labels incorporated either in DPPC bilayers (dotted line) or DPPC bilayers containing Losartan at mole fraction $x = 0.10$ (solid line) obtained at the temperatures indicated, over the range 20–43°C. Total scan width = 100 G.

both in the gel and in the fluid phase ($\Delta A_{max}/(A_{max}-A_0) = 31.5\%$ as opposed to 10.7%, and 11.9% as opposed to 10.1% at 20°C and 43°C, respectively).

At yet higher concentration of the antagonist $x = 0.60$ (Fig. 7B), the effects of the drug are qualitatively similar to those described above for mole fraction $x = 0.40$. Quantitatively, the effects are correspondingly larger. The 5-PCSL spin label is restricted in motion in both the gel and fluid phases, showing greater increases in outer hyperfine splitting $2A_{max}$ than at drug mole fraction $x = 0.40$ ($\Delta A_{max}/(A_{max}-A_0) = 6.3\%$ as opposed to 5.3%, and 5.3% as opposed to 3.9% at 30°C and 43°C, respectively). In the case of the 14-PCSL spin labels, a greater restriction in their motion is observed in the gel phase, compared to the corresponding one for $x = 0.40$ ($\Delta A_{max}/(A_{max}-A_0) = 34.8\%$ as opposed to 31.5% at 20°C). In the liquid–crystalline phase, the restriction in motion of the spin labels incorporated in the preparation containing the antagonist is smaller compared to the corresponding one that is observed in the case of mole fraction $x = 0.40$ ($\Delta A_{max}/(A_{max}-A_0) = 6.0\%$ as compared with 11.9% at 43°C).

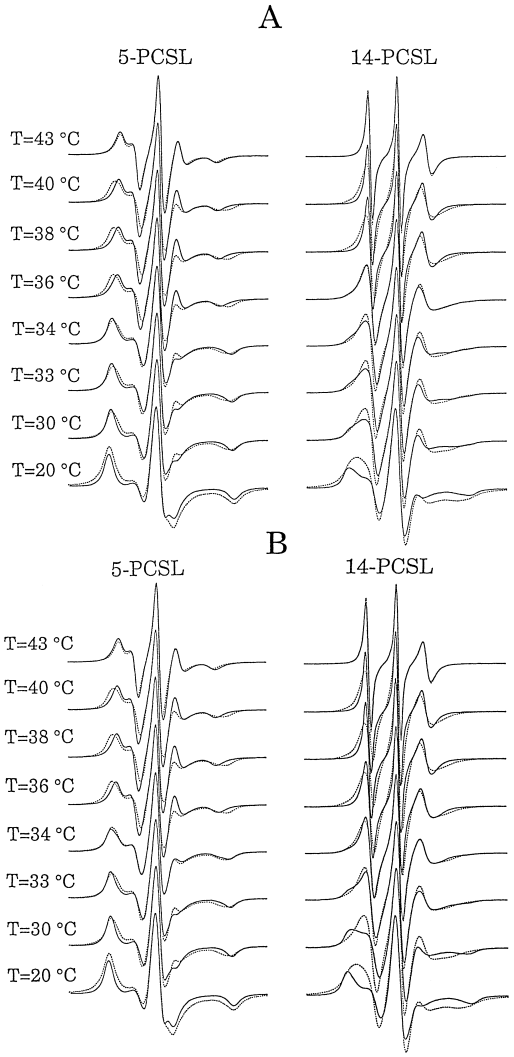

Fig. 7. ESR spectra of 5- and 14-PCSL spin labels incorporated either in DPPC bilayers (dotted line) or DPPC bilayers containing Losartan (solid line) at mole fraction $x = 0.40$ (A) or $x = 0.60$ (B) obtained at the temperatures indicated, over the range 20–43°C. Total scan width = 100 G.

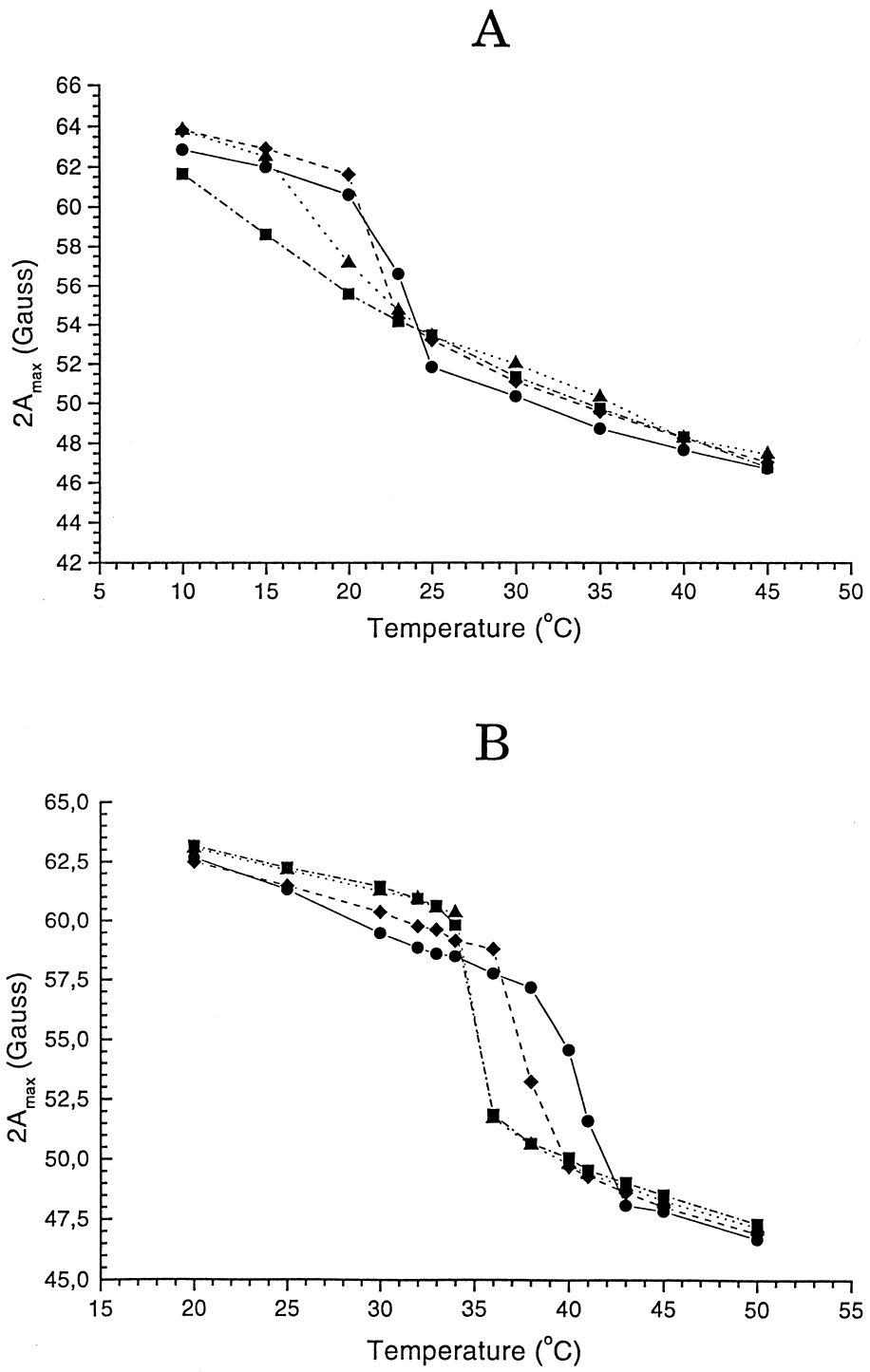

Fig. 8. Temperature dependence of the outer hyperfine splitting ($2A_{max}$) of 5-PCSL spin label incorporated into DMPC (A) or DPPC (B) bilayers containing Losartan at different concentrations. ●, Pure phospholipid bilayers; ♦, bilayers containing Losartan at mole fraction $x = 0.10$; ▲, bilayers containing Losartan at mole fraction $x = 0.40$; and ■, bilayers containing Losartan at mole fraction $x = 0.60$.

Fig. 8 shows the temperature dependence of the outer hyperfine splittings $2A_{max}$, in the ESR spectra of 5-PCSL spin label incorporated into DMPC (Fig. 8A) or DPPC (Fig. 8B) bilayers, in the absence and presence of different concentrations of Losartan. It is observed that the incorporation of the antagonist at increasing mole fractions in DMPC or DPPC bilayers causes a progressive shift of the corresponding chain-melting transitions to lower temperatures. Additionally, in DMPC bilayers, the observed transition is broadened significantly due to the incorporation of Losartan at mole fractions $\geq x = 0.40$. These results are in agreement with our DSC data (Figs. 2 and 3). Furthermore, in Fig. 8 is shown that below and above the observed chain-melting transition, the values of $2A_{max}$ in the ESR spectra of 5-PCSL spin label incorporated into DMPC or DPPC bilayers containing the antagonist are larger than the corresponding ones in the case of pure phospholipid bilayers. This indicates a restriction in the spin label motion that is caused by the presence of Losartan in the membranes. The only exception is the preparation of DMPC bilayers containing Losartan at mole fraction $x = 0.60$, which in the gel phase shows decreased $2A_{max}$ value compared to that of DMPC alone. In this case, the observed chain-melting transition is significantly broadened and shifted to low temperatures and as a result, no restriction in the motion of 5-PCSL spin labels is observed.

4. Discussion

The high incidence of cardiovascular diseases, for example hypertension and heart failure, has generated an enormous interest associated with the discovery of non-peptide AT_1 receptor antagonists of ANG II, such as Losartan, an orally active antagonist which has received FDA approval as a new drug.

The membrane lipid bilayer serves as a structural and dynamic matrix into which numerous functional proteins are embedded. A lipophilic drug may produce some of its effects by perturbing the lipid bilayer. This event, which is manifested as a change in membrane dynamics, may modulate the function of one or more membrane-associated proteins [30–35]. The elucidation of drug interactions with membranes and their effects on membrane organization in relation to their membrane-involving functions is a recent approach to molecular-level mechanisms of action (or side effects) of the drugs. Besides its action on AT_1 and AT_2 receptors, it is known that ANG II also interacts with the lipid bilayer of biological membranes. Our study was aimed to investigate Losartan's interactions with the phospholipid bilayer component of membranes. The present study is also essential due to the knowledge that the major determinants of Losartan binding appear to be residues located within the transmembrane regions III, IV, V, VI and VII of the AT_1 receptor [36].

Due to the complexity of membrane dynamics and thermodynamics, a single biophysical method is not sufficient for a detailed analysis of their properties. A combination of DSC and ESR spectroscopy, two biophysical techniques that can give complementary information about the thermotropic and dynamic properties of biologically active molecules in membranes, was used.

Our DSC results show that Losartan has a very marked effect on the cooperativity in thermodynamic properties of DMPC and DPPC bilayers. This is especially dramatic with DMPC bilayers, for which the thermotropic transition region is very extensively broadened and displays a complex endothermic behavior characterized by three major calorimetric peaks (Fig. 2). Both the size of the total calorimetric enthalpy and the correlation with the temperature dependence of the spin-label ESR spectra indicate that this thermotropic behavior is associated with melting of the lipid chains. The complex endotherms of DMPC at high Losartan concentration bear a strong similarity to those found for the anionic lipid dimyristoylphosphatidylglycerol at low ionic strength [37]. In the latter case, a broad thermotropic transition extending from 20 to 28°C is observed, with two outer calorimetric peaks and a third broader intermediate calorimetric peak. Most significantly, this was accompanied by a large increase in the relative viscosity of the lipid dispersion in the transition region, just as is observed here. Recently, it has been demonstrated that this corresponds to a reversible transition from a vesicular suspension to an extended bilayer network in the chain-melting region [38]. Solvent-associated or interfacial interactions favoring a change in membrane curvature were proposed to explain this behavior. The strongly enhanced bending

elasticity arising from lipid-state coexistence in the chain-melting region is then sufficient to induce the structural transition. It was further demonstrated that the complex thermotropic behavior involving three calorimetric peaks is an inevitable thermodynamic consequence of the structural transition. Almost certainly, the same situation holds here with DMPC and high concentrations of Losartan. Further evidence, in addition to the diagnostic calorimetric fingerprint and viscosity anomaly, comes from the strong lipid chainlength dependence. Only a broadened chain-melting transition, without the characteristic three-peak structure is found for dipalmitoylphosphatidylglycerol at low ionic strength [38], exactly as found here for DPPC of the same chainlength, at high concentrations of Losartan. A more limited DSC study published previously [39] is qualitatively consistent with the present studies. Any quantitative differences are probably attributable to the different experimental conditions used. In particular, different water-to-lipid ratios are expected to affect the degree of partitioning of Losartan into the membrane.

ESR spectroscopy was performed to study the effect of Losartan on the chain motion and packing in bilayer membranes by using different positional isomers of phosphatidylcholine (*n*-PCSL) spin probes incorporated in DMPC or DPPC bilayers. The ESR spectra obtained showed that Losartan shifted the chain-melting phase transition to lower temperatures and broadened the region of phase coexistence, in agreement with DSC data (Figs. 2 and 3).

A decrease in chain mobility of phosphatidylcholine bilayers is induced by the incorporation of antagonist, both in the gel and fluid phases of the membrane (Figs. 4–7). In the fluid phase, this change was registered more sensitively by the spin label on the 5-position than by spin labels positioned further down the chain. This could indicate that Losartan is located closer to the interfacial region than to the hydrophobic core of the phosphatidylcholine bilayer. Interestingly, Losartan improves the solubility of the lipid probes, and possibly of other components, in the gel phase of phosphatidylcholine bilayers. This is seen from the reduction in spin–spin interactions between the 12-PCSL probes and suggests that incorporation of Losartan relaxes the tight chain packing in the gel phase, even though the segmental mobility is reduced. Increasing the concentration of Losartan to $x = 0.40$, progressively decreases the chain mobility in the DMPC and DPPC gel and fluid phases (Figs. 5 and 7). At a very high mole fraction ($x = 0.60$) in DMPC bilayers, the effects of antagonist differ qualitatively from those at lower molar fractions. The chain segmental mobility is then increased in the gel phase of DMPC, and the decrease in chain mobility registered by 5-PCSL in the fluid phase is smaller, whereas that registered by 14-PCSL is further increased. This indicates a different mode of interaction with Losartan, possibly arising from a location deeper within the bilayer at high mole fractions of antagonist.

Our results, as far as we know, are one of the first studies of the interactions of non-peptide ANG II AT_1 receptor antagonists with phospholipid membranes. Using DSC, we have shown that Losartan is able to intercalate in phosphatidylcholine bilayers. ESR spectroscopy showed that one possible mode of insertion is location of Losartan's polar hydroxyl group near the carbonyl groups of the phospholipids, with which it could form hydrogen bonds. Then the butyl chain of the antagonist could be extended in the hydrophobic region of the bilayer and the tetrazole group located near the phosphate of the phospholipid headgroups. This hypothesis is supported by the finding that the greater effect of the antagonist on acyl chain mobility of the spin probes was observed in the spectra of 5-PCSLs. A similar membrane location was also proposed for classical cannabinoid molecules possessing a phenolic hydroxyl group [18,21,40,41]. At high drug concentrations, it seems that Losartan inserts deeper in the bilayers, affecting more strongly the mobility of 14-PCSL spin probes for DMPC and DPPC membranes while the mobility of 5-PCSL is less affected. This may be caused by self-association of Losartan with intermolecular bonding of its polar hydroxyl groups that results in a more hydrophobic behavior of the antagonist. A similar effect was observed with cyclosporin incorporated in dielaidoylphosphatidylethanolamine membranes [42].

In conclusion, our study has shown that Losartan interacts with phospholipid membranes by affecting both their thermotropic behavior and molecular mobility. Particularly striking is the effect that high concentration of Losartan has on the chain-melting be-

havior of DMPC and the associated changes in vesicular structure that are induced in this region. These results must be taken into account in the pharmacological application of the antagonist and open up the possibility that Losartan, besides its antagonist action on the receptor level, could exert some of its effects through its interaction with the lipids of the membrane bilayer.

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